

A Laminin-Collagen Complex Drives Human Epidermal Carcinogenesis through Phosphoinositol-3-Kinase Activation

Elizabeth A. Waterman, Noriyasu Sakai, Ngon T. Nguyen, Basil A.J. Horst, Dallas P. Veitch, Clara N. Dey, Susana Ortiz-Urda, Paul A. Khavari, and M. Peter Marinkovich

Dermatology Service, Palo Alto VAHCS, Palo Alto, California and Program in Epithelial Biology, Stanford University School of Medicine, Stanford, California

Abstract

Laminin-332 (formerly laminin-5) and collagen VII are basement membrane proteins expressed at the invasive front of human squamous cell carcinoma (SCC) tumors. These proteins have protumorigenic properties, but whether laminin-332 and collagen VII promote SCC tumors by providing adhesion or other nonadhesive extracellular cues, or whether laminin-332 and collagen VII interact together in this process remains unknown. In this study, we examined the role of these molecules by a structural approach using an *in vivo* model of human SCC tumorigenesis. Here, we show that individual domains (VI and V-III) on the laminin-332 $\beta 3$ chain provide distinct and highly divergent cell adhesion and tumor-promoting functions. We found that laminin $\beta 3$ domain VI provided a critical role in the assembly of stable adhesion complexes, but this domain was not required in SCC tumors. Instead, we found that laminin $\beta 3$ domain V-III played an essential role in SCC carcinogenesis/invasion through binding to collagen VII, which in turn, led to phosphoinositol-3-kinase activation and protection from apoptosis. Overexpression of constitutively active p110 phosphoinositol-3-kinase subunit was sufficient to restore invasion and tumorigenesis in transformed cells lacking laminin-332/collagen VII interaction in a manner independent of cellular adhesion. These studies show distinctive adhesive and signaling functions in individual domains of laminin-332, one which is required for normal epithelial adhesion and one which is required for SCC tumorigenesis. This uncoupling of stable adhesion from tumor progression in our studies suggests that laminin-332/collagen VII interaction promotes epidermal carcinogenesis through signaling rather than adhesion. [Cancer Res 2007;67(9):4264–70]

Introduction

Squamous cell carcinoma (SCC), the second most common human cancer (1), is highly invasive and can progress to lethal metastases. SCC tumors deposit the heterotrimeric basement membrane zone molecule laminin-332 (previously known as laminin-5) at their stromal interface (2). Laminin-332 expression correlates with tumor invasiveness and overall patient prognosis in a wide spectrum of SCCs (3). Laminin-332 facilitates epithelial

adhesion in normal tissues (4) by linking integrin-containing adhesive structures, termed hemidesmosomes, with collagen VII in the basement membrane zone (5, 6).

Recent studies have shown that s.c. introduction of primary human keratinocytes coexpressing oncogenic Ras and inhibitor of $\kappa B\alpha$ ($I\kappa B\alpha$) to immunodeficient mice produces tumors which are histologically and biochemically indistinguishable from human SCC (7). This model has been used to show, through both antibody inhibition and genetic deletion/restoration studies, that laminin-332 and collagen VII are each required for SCC tumorigenesis (7, 8). These studies raise questions as to whether the functions of laminin-332 and collagen VII in SCC tumors may be linked or require the interaction of these two molecules. It has been suggested that these molecules promote SCC tumorigenesis through adhesion or anchoring (9); however, how laminin-332 and collagen VII actually support SCC development remains unknown.

In this study, we addressed how laminin-332 and collagen VII promote SCC tumorigenesis, and whether direct interaction of these molecules was required. Laminin-332 contains $\alpha 3$, $\beta 3$, and $\gamma 2$ chains, with the $\alpha 3$ and $\gamma 2$ chains undergoing proteolytic processing (10). Domains I/II on each chain mediate intramolecular trimeric assembly through α -helix formation, whereas the other globular domains are involved with extramolecular associations (11). As previous studies suggested that laminin-332 $\beta 3$ chain globular domains contain the collagen VII binding site (12, 13), we engineered and expressed laminin-332 $\beta 3$ mutants containing deletions of these domains in human junctional epidermolysis bullosa (JEB) patient keratinocytes lacking endogenous expression of laminin $\beta 3$ chain due to *LAMB3* gene mutations (7). We did *in vitro* and *in vivo* studies to examine the oncogenic potential of these mutants.

Materials and Methods

Cell culture. Primary keratinocytes isolated from normal skin, and patients with junctional and recessive dystrophic epidermolysis bullosa (RDEB) lacking laminin-332 and collagen VII expression, respectively, have been previously characterized (7, 8). Keratinocytes were cultured in defined keratinocyte serum-free medium (SFM; Life Technologies) at 37°C in a humidified 5% CO₂ incubator. Modified human 293 Phoenix cells (a gift from Dr. G. Nolan, Stanford, CA) were cultured in DMEM supplemented with 10% FCS, 100 IU/mL penicillin, and 100 μ g/mL streptomycin at 37°C in a humidified 10% CO₂ incubator.

Antibodies. Antibodies were obtained from the following sources: $\alpha 6$ integrin antibody G0H3, $\beta 4$ integrin antibody ASC-8 used for attachment assays, and β -actin (Sigma); $\beta 4$ integrin antibody 3E1 used for confocal microscopy and $\beta 1$ integrin antibody P5D2 (Chemicon); p-AKT and total AKT (Cell Signaling Technologies); $I\kappa B\alpha$ (Calbiochem); H-Ras (Santa Cruz Biotechnology); paxillin antibody 165 (BD Biosciences); Alexa Fluor 350-conjugated phalloidin (Invitrogen); TRITC-conjugated goat anti-rabbit,

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

Competing interests statement: The authors declare that they have no competing financial interests.

Requests for reprints: M. Peter Marinkovich, Program in Epithelial Biology, Stanford University School of Medicine, 269 Campus Drive, Room 2145, Stanford, CA 94305-5148. Phone: 650-498-5425; Fax: 650-723-8762; E-mail: mpm@stanford.edu.

©2007 American Association for Cancer Research.
doi:10.1158/0008-5472.CAN-06-4141

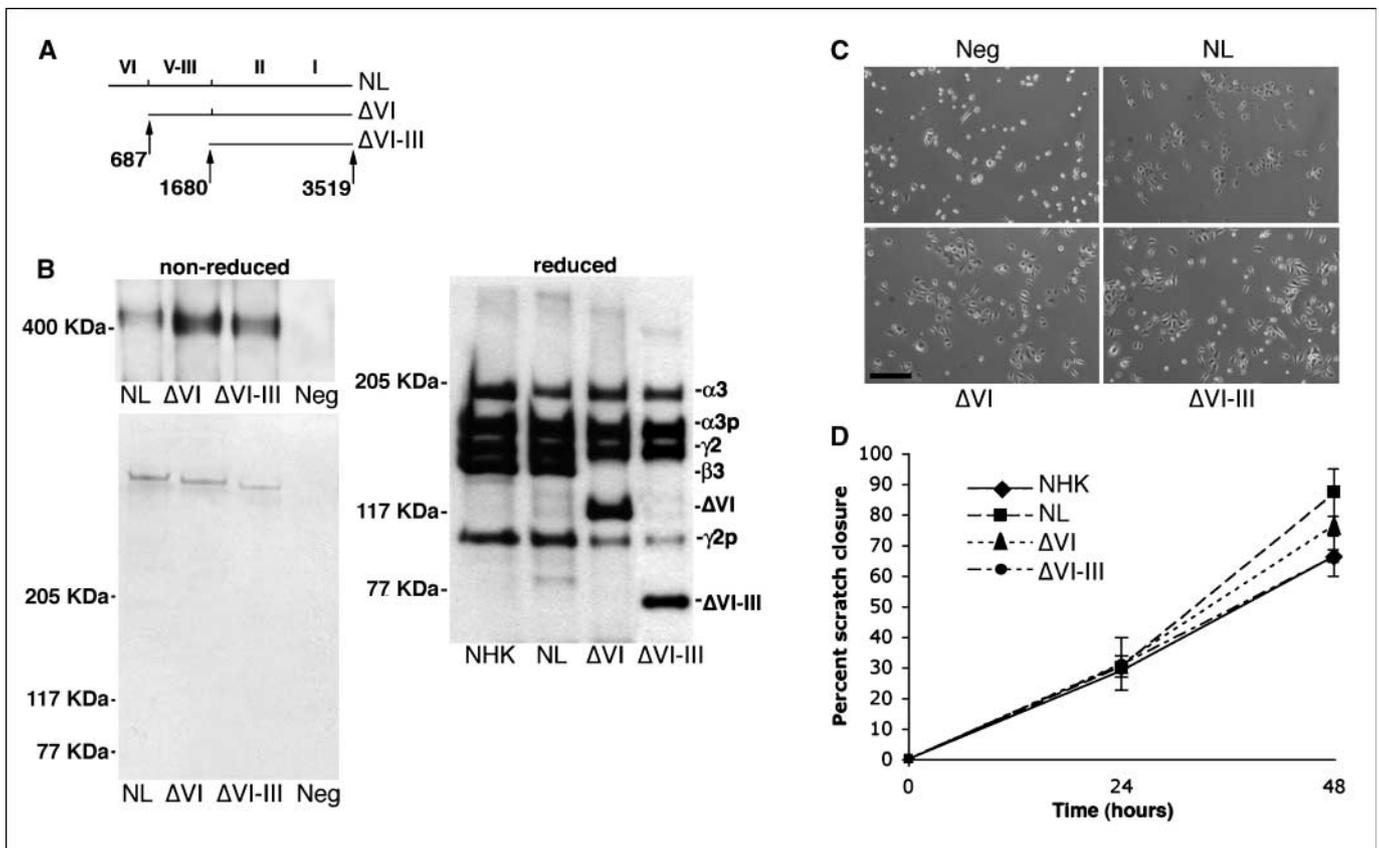


Figure 1. Mutant laminin-332 expression and function in human keratinocytes. *A*, full-length normal laminin (NL) $\beta 3$ chain domain structure and ΔVI and $\Delta VI-III$ mutant start and stop sites (*LAMB3* gene base pairs). *B*, analysis of trimeric laminin-332 purified from the medium of retrovirally transduced JEB keratinocytes expressing full-length $\beta 3$ and therefore normal laminin-332 (NL), ΔVI , and $\Delta VI-III$ laminin-332, or negative control JEB keratinocytes transduced with LacZ vector. *Top left*, immunoblot of nonreduced protein using anti-laminin-332 antibody; *bottom left*, Coomassie blue total protein staining. Molecular weight markers are shown in kilodaltons (kDa). *Right*, reduced immunoblot analysis of matrix from normal human keratinocytes (NHK) and keratinocytes described above using anti-laminin-332 antibody. Markers indicate the positions of the following laminin chains: $\alpha 3$, processed $\alpha 3$ ($\alpha 3p$), $\gamma 2$, $\beta 3$, ΔVI , processed $\gamma 2$ ($\gamma 2p$), and $\Delta VI-III$. *C*, phase contrast microscopy of keratinocytes described above (bar, 100 μm). *D*, migration of NHK and keratinocytes described above as measured by scratch assay over the times indicated.

Cy5-conjugated goat anti-rat, and FITC-conjugated donkey anti-mouse secondary antibodies (Jackson ImmunoResearch); sheep anti-mouse and donkey anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Amersham); anti-plectin antibody HD121 (ref. 14; a gift from Dr. Katsushi Owaribe, Nagoya, Japan) and anti-CD151 antibody, 5C11 (ref. 15; a gift from Martin Hemler, Boston, MA). Laminin-332 rabbit polyclonal antibody (10) and monoclonal antibody BM165 used for immunoaffinity purification (16) were described previously.

Complementary DNA constructs. Full-length human laminin-332 $\beta 3$ chain cDNA (HuLAMB3) has been previously characterized (17). Two cDNAs encoding HuLAMB3 with NH₂-terminal deletions comprising either domain VI (ΔVI), or domains III/V and VI ($\Delta VI-III$), respectively, were generated by PCR, verified by direct sequence analysis and cloned into the retroviral vector backbone LZRS (18) containing the encephalomyocarditis virus-IRES and blasticidin resistance sequences (19) and a Gateway (Invitrogen) destination site (pLZRS-GATEWAY). The BM40 signal sequence, which has previously been shown to facilitate the secretion of extracellular matrix proteins (20), was incorporated upstream and in-frame of the mutant HuLAMB3 for directing expression. For further details of the constructs and the sequences of PCR primers used, see Supplementary Information.

Retroviral expression vectors encoding either Ha-Ras, I κ B α M (7), or active phosphoinositol-3-kinase (PI3K) p110-CAAX (21) have been previously characterized. Amphotropic retroviral supernatant production (18) and retroviral keratinocyte transduction were done as previously described (19).

Microscopy. Tumor samples were fixed in 4% paraformaldehyde and paraffin embedded. Four-micron sections were stained with H&E according to standard procedures. A terminal nucleotidyl transferase-mediated nick end labeling (TUNEL) assay was done using the ApopTag peroxidase *in situ* apoptosis detection kit according to the manufacturer's instructions (Chemicon). Light and immunofluorescent microscopy sections were visualized and photographed using a Zeiss Axiovert 100 inverted microscope.

For visualization of keratinocyte attachment complexes, confocal microscopy was done using a Zeiss LSM 510 confocal laser scanning microscope equipped with a coherent Mira 900 tunable titanium/sapphire laser for two-photon excitation. Cells were cultured in HAMF12/DMEM (1:3) containing 10% FCS, 0.4 $\mu g/mL$ of hydrocortisone, and 10^{-6} mol/L of isoproterenol (both from Sigma) for 24 h. Cells were then fixed with 3% paraformaldehyde/0.5% Triton X-100 in PBS at room temperature for 30 min and blocked with 1% bovine serum albumin for 60 min before staining with appropriate primary and secondary antibodies. In some instances, Hoechst stain was applied to visualize the nuclei.

Protein analysis. Cellular proteins (10) and keratinocyte matrix (22) were extracted and analyzed by immunoblot as previously described. Laminin-332 was immunoaffinity-purified from conditioned keratinocyte medium as previously described (16); briefly, conditioned medium from keratinocytes expressing normal or mutant laminin-332 was passed over a monoclonal antibody BM165-sepharose column, washed extensively, eluted with 0.2 mol/L of glycine (pH 4), dialyzed extensively against PBS, analyzed by immunoblot and Coomassie blue total protein staining and quantified by

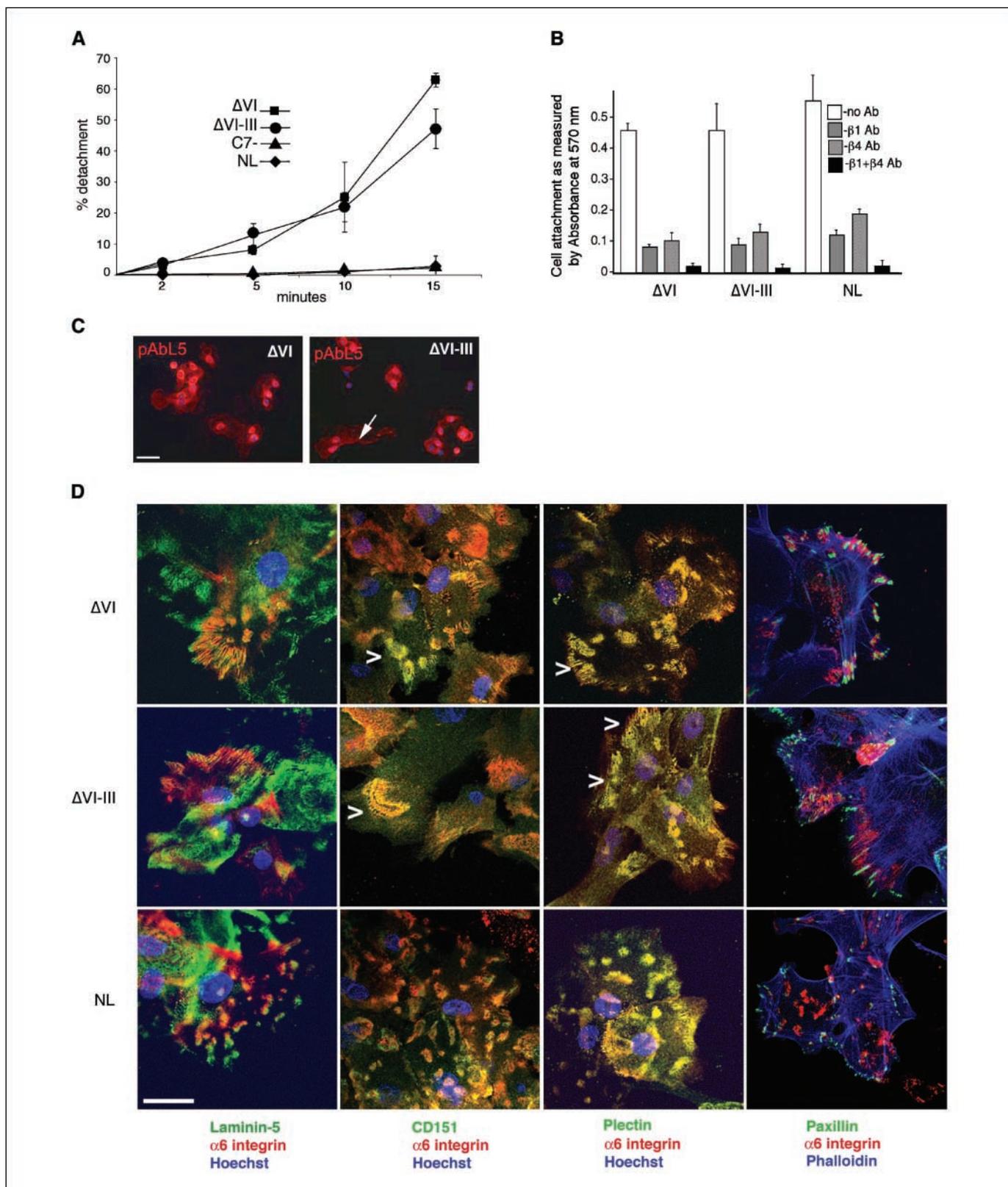


Figure 2. Laminin domain VI promotes keratinocyte adhesion. *A*, analysis of SA from keratinocytes expressing Δ VI, Δ VI-III, or normal laminin-332 (NL) or collagen null keratinocytes (C7-). The percentage of cells detached by dilute trypsin was measured over the times indicated. *B*, short-term attachment of normal human keratinocytes to purified Δ VI, Δ VI-III, and normal laminin-332 (NL)-coated wells, in the presence of indicated integrin-blocking antibodies. *C*, laminin-332 deposition by Δ VI and Δ VI-III mutant keratinocytes assessed by indirect immunofluorescent microscopy. Note trails of laminin-332 adjacent to cells (arrow); pAbL5, polyclonal anti-laminin-332 antibody (bar, 50 μ m). *D*, confocal microscopic analysis of adhesion complexes produced by keratinocytes expressing Δ VI, Δ VI-III, and normal laminin-332 (NL) by using the indicated antibodies with secondary antibodies or nuclear (Hoechst) stain indicated by the colors. Note that Δ VI and Δ VI-III keratinocytes expressed abnormally large peripheral SAs (arrows), which colocalized with paxillin and actin microfilaments (bar, 100 μ m).

total protein assay (Bio-Rad; ref. 22) prior to use in attachment and ligand binding assays. p-AKT activity was done on cells plated on Matrigel-coated dishes (23) under standard culture conditions and was quantified using a Bio-Rad GS-710-calibrated imaging densitometer using triplicate samples and data were normalized to β -actin obtained from the cell samples. Solid-phase binding assays were carried out as previously described (12). Purified collagen VII NC1 domain (8) was used as the immobilized ligand at a concentration of 50 nmol/L. Normal, Δ VI, or Δ VI-III laminin-332 was used as the second protein at a range between 0 and 100 nmol/L. Rabbit polyclonal laminin-332 antibody was used as the primary antibody with rabbit-horseradish peroxidase secondary antibody (Amersham).

Cell adhesion, migration, and invasion assays. Cell detachment assays were carried out as previously described (24); briefly, 2×10^4 cells/cm² were incubated for 24 h at 50% confluence. Monolayers were washed and detached cells were quantified at increasing time intervals after incubation in a 1:70 dilution of trypsin/EDTA in PBS (Bio Whittaker). Each experiment was done in triplicate. Short-term (30 min) cell attachment assays to dishes coated with purified laminin-332 (2 μ g/well) in the presence of 15 μ g/mL of integrin-inhibiting antibodies was done as previously described (25). Binding of normal keratinocytes to uncoated dishes was found to be <1% of the binding observed on normal laminin-332-coated dishes after the 30 min assay (data not shown). Cell monolayer scratch assays (26) were done by plating 10^6 cells into 60 mm tissue culture plates and incubating cells in SFM for 24 h. Media was changed to SFM/WA for 16 h. Fresh mitomycin-C (Sigma) was added at 10 μ g/mL and cells were incubated for 3 h on ice. Cells were washed twice with SFM/WA and scratched with a 1-mm cell scraper. Plates were washed thrice with SFM/WA and marked areas photographed using a Zeiss Axiovert 25 microscope (50 \times magnification). Migration was quantified by calculating the percentage of change in the area between migrating cell sheets using NIH image software and more than three repeats per data point. The *in vitro* invasion assays (8) were done as previously described; briefly, assays were done in triplicate using chambers containing a polycarbonate member with 8- μ m pores, coated with Matrigel (Becton Dickinson). After 24 h, invasive cells in the bottom chamber were lysed and quantified using CyQuant GR dye. Data represented the results from triplicate independent experiments and was quantified as a percentage of basal invasion by JEB (laminin-332 null) cells alone.

Tumorigenicity assay. The ability of JEB patient cells expressing mutant laminin-332 to form SCC-like tumors was assessed by tumorigenicity assays as previously described (7). Briefly, keratinocytes were subjected to overexpression of oncogenic Ras and blockade of nuclear factor κ B by dual infection with retroviral titer produced from modified 293 cells transfected with either LZRS-IRES-blasticidin/Ha-Ras or LZRS-IRES-blasticidin/ κ B α M. Gene transfer was verified by immunoblotting of cell lysates. One million Ras/ κ B α M-transformed cells suspended in 200 μ L of Matrigel (Becton Dickinson) were injected s.c. to the dorsal flank of 6-week-old nude mice, five mice were used for each condition, including Δ VI, Δ VI-III, normal, and Δ VI-III + PI3K conditions. Tumor volume was measured weekly for a total of 5 weeks. All animal studies were conducted in accord with protocols approved by the Stanford Animal Use Committee.

Results

Mutant laminin-332 expression and function in human keratinocytes. Two laminin β 3 chain mutants were engineered, one contained an NH₂-terminal deletion of domain VI (Δ VI) and another contained a deletion of domains VI/V-III (Δ VI-III) (Fig. 1A). Retroviral transfer of Δ VI and Δ VI-III laminin β 3 mutants to β 3 null JEB keratinocytes restored the assembly and secretion of laminin-332 trimers into the conditioned medium (Fig. 1B), which was immunoaffinity-purified and analyzed by total protein staining (Fig. 1B). Δ VI and Δ VI-III β 3 laminin chains of predicted apparent molecular weight were deposited into the extracellular matrix at levels similar to the normal laminin β 3 chain (Fig. 1B). The processing of the laminin γ 2 chain was decreased in each of the mutant cells. Together, these results suggest that domains VI and V-III of laminin β 3 were not required either for laminin-332 assembly, secretion, or matrix deposition. Δ VI- and Δ VI-III-expressing keratinocytes exhibited spreading and flattening when cultured on tissue culture plastic in contrast to the rounded appearance of laminin-332 null (LacZ) control cells (Fig. 1C). Δ VI and Δ VI-III cells migrated equivalently in scratch assays compared with cells expressing normal laminin-332. These

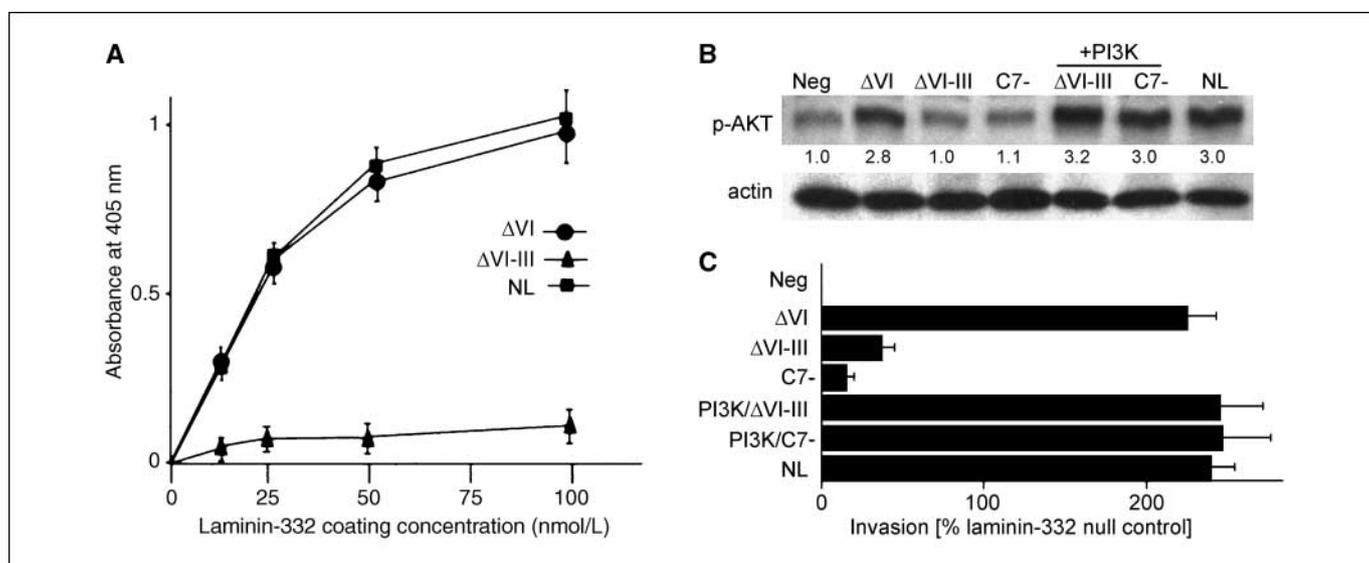


Figure 3. Carcinoma invasion and PI3K pathway activation correlates with laminin-332/collagen VII interaction. *A*, interaction of purified normal (NL), Δ VI, and Δ VI-III laminin-332 with collagen VII NC1 domain by solid phase ligand binding assay. *B*, Ras/ κ B α -transformed keratinocytes expressing no laminin-332 (LacZ), Δ VI, Δ VI-III, or normal laminin-332 (NL), as well as collagen VII null RDEB keratinocytes (C7-) were analyzed by immunoblot using a p-AKT antibody. Note that cells with lack of collagen VII/laminin-332 interaction (Δ VI-III and C7-) showed reduced p-AKT, which could be restored by overexpression of p110 PI3K subunit cDNA (PK). *C*, keratinocytes expressing Δ VI, Δ VI-III, or normal laminin-332 (NL), or collagen VII null RDEB keratinocytes (C7-) were Ras/ κ B α -transformed and analyzed by *in vitro* invasion assay. Note that only cells with lack of collagen VII/laminin-332 interaction (Δ VI-III and C7-) showed deficient invasion which was restored with p110 overexpression (PK).

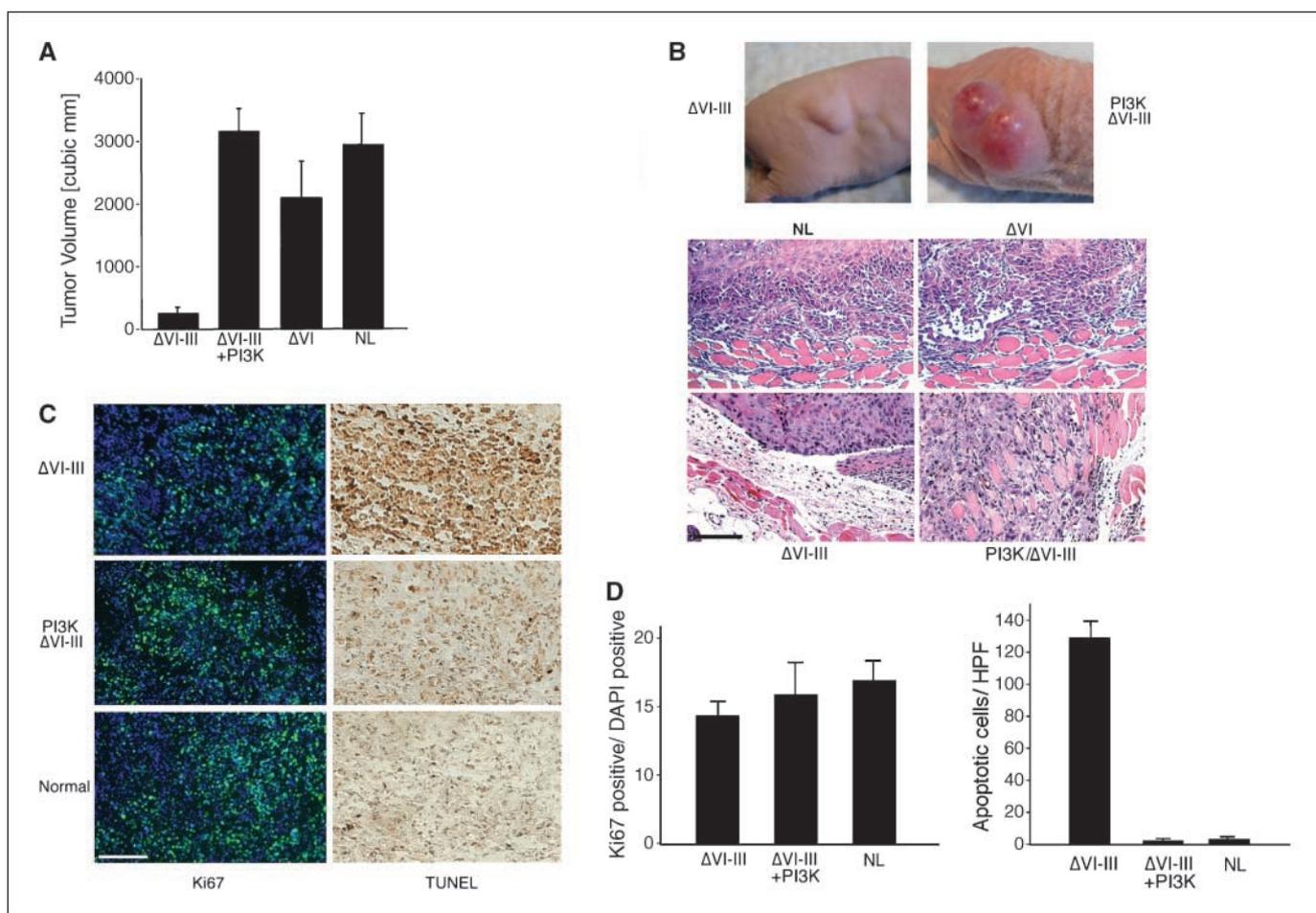


Figure 4. Epidermal tumorigenesis and PI3K pathway activation is associated with collagen VII/laminin-332 interaction. **A**, analysis of tumor formation in nude mice after injection of Ras/IKB-transformed JEB keratinocytes expressing Δ VI, Δ VI-III, or normal laminin-332 (NL). Note a lack of tumor formation by transformed VI-III cells, which could be restored to normal levels with the introduction of p110 cDNA (PK). **B**, typical clinical appearance of tumors 5 wks after injection of Δ VI-III expressing or Δ VI-III/PI3K expressing Ras/IKB-transformed keratinocytes (top). Histology of deep aspects of 5-wk-old tumors produced by Ras/IKB-transformed keratinocytes expressing normal, Δ VI, or Δ VI-III laminin-332 (bottom). Note the lack of underlying muscle invasion in the Δ VI-III condition, which was restored by p110 (PI3K) expression (bar, 100 μ m). **C**, TUNEL assay and Ki67 expression in Δ VI-III, Δ VI-III/PI3K, and normal (NL) tumors (bar, 50 μ m) with quantification (**D**).

results suggest that laminin β 3 domains VI and V-III are not required for keratinocyte spreading or migration (Fig. 1D).

Laminin β 3 domain VI promotes keratinocyte adhesion.

Despite other normal properties, Δ VI and Δ VI-III cells showed marked adhesion defects. Δ VI and Δ VI-III cells showed a remarkable sensitivity to trypsin-induced detachment compared with normal cells (Fig. 2A). Purified Δ VI and Δ VI-III laminins, coated onto cell culture surfaces, supported α 3 β 1 and α 6 β 4 integrin-dependent cell attachment in a manner similar to purified normal laminin-332 (Fig. 2B), suggesting that the adhesion abnormality was not related to the altered availability of integrin-binding sites on the mutant laminin molecules. Decreased laminin γ 2 chain processing was associated with increased keratinocyte adhesion (24), and as such, cannot explain the decreased adhesion in our mutants. Also, this adhesion abnormality seemed to be distinct from any dependence on collagen VII, as collagen VII null human RDEB keratinocytes showed a level of resistance to trypsin dissociation similar to normal cells (Fig. 2A). Cells expressing mutant laminins showed *in vivo* growth comparable to keratinocytes expressing normal laminin-332 (Supplementary Fig. S2).

Laminin-332 normally assembles with other hemidesmosome components to form attachment structures termed stable adhe-

sions (SA; ref. 27). Although Δ VI and Δ VI-III keratinocytes deposited laminin-332 into their extracellular periphery and behind them in migratory trails (Fig. 2C, arrow) similar to normal keratinocytes (16), both Δ VI and Δ VI-III mutants produced strikingly abnormal SAs. SAs in Δ VI and Δ VI-III mutant cells distributed peripherally compared with the central SA distribution of normal cells (Fig. 2D). Moreover, Δ VI and Δ VI-III SAs showed linear discontinuities which we noted were associated with the abnormal actin insertion into SAs. Δ VI and Δ VI-III SAs also contained abnormal collections of focal adhesion components including paxillin (Fig. 2D) and α 3 β 1 integrin (data not shown). Although focal adhesions are believed to transition into hemidesmosomes (28), this transition seems to be incomplete in Δ VI and Δ VI-III cells, which could explain the resultant adhesion abnormality. The equivalent functional and morphologic adhesion abnormalities in either Δ VI or Δ VI-III mutants indicate that the underlying defect in each cell type results from a lack of domain VI. Thus, laminin β 3 domain VI may promote adhesion by facilitating focal adhesion/hemidesmosome transition in keratinocytes without altering integrin binding sites on the laminin-332 molecule.

Carcinoma invasion and PI3K pathway activation correlates with laminin-332/collagen VII interaction. Purified Δ VI and

Δ VI-III, and normal laminin-332 showed vastly divergent collagen VII binding properties, seen via solid phase ligand binding assay (Fig. 3A). Δ VI laminin-332 bound collagen VII NC1 domain in a normal, dose-dependent manner; however, β 3 Δ VI-III laminin-332 showed little or no binding. These results were consistent with previous studies suggesting that the laminin β 3 chain mediates the binding of laminin-332 to collagen VII (12, 13). However, the presence of collagen VII/laminin-332 binding did not promote increased SA in Δ VI cells compared with Δ VI-III cells (Fig. 2A). Moreover, collagen VII null cells showed no adhesion abnormalities in our assay. Therefore, we were unable to establish a link between collagen VII/laminin-332 interaction and cell adhesion.

Previous studies have linked laminin/integrin interactions with PI3K activation in several carcinoma cell lines (29). These studies differed considerably from ours in that the cell lines studied were not SCC-derived and the laminin studied was laminin-111, which does not interact with collagen VII. However, even though deletions of the laminin β 3 chain seemed to have no effect on laminin-integrin binding, we wanted to explore the possibility that interactions of laminins with molecules other than integrins could also be associated with PI3K activation and/or tumor invasion.

Towards this end, we sought to correlate collagen VII/laminin-332 binding with PI3K activation and SCC invasion. To address this further, we transformed keratinocytes expressing Δ VI, Δ VI-III, normal laminin-332, or laminin-332 negative keratinocytes (Neg), although retroviral expression of Ras and $I\kappa B\alpha$ in an approach established to directly convert these cells into epidermal SCC (7, 8). After transformation, we examined the activity of the PI3K pathway using a phosphorylated AKT antibody by immunoblot. We found that transformed Δ VI-III cells showed a striking reduction in the levels of p-AKT, reflective of PI3K pathway activation, compared with Δ VI or normal cells (Fig. 3B). Interestingly, transformed collagen VII null RDEB keratinocytes showed a similar lack of p-AKT. This reduction in transformed Δ VI-III and collagen VII null cells was restored to normal levels through overexpression of the constitutively active PI3K p110 α subunit. We next tested the invasive properties of each of these transformed cell types *in vitro* (Fig. 3C). We found that Δ VI-III and collagen VII null cells showed a significant lack of invasion compared with Δ VI or normal cells. However, the lack of invasion in Δ VI-III and collagen VII null cells could be restored to normal levels through PI3K p110 α subunit overexpression. It is known that p110 overexpression reduces the proliferative rate of normal untransformed keratinocytes (30), which we also noted (data not shown). These results showed a clear association between the binding of collagen VII to laminin-332, activation of the PI3K signaling pathway and cellular invasion.

Epidermal tumorigenesis and PI3K pathway activation is associated with collagen VII/laminin-332 interaction. After injection to immunodeficient mice, transformed Δ VI-III cells showed a significant lack of tumor formation compared with Δ VI or normal cells; however, tumor formation could be restored in transformed Δ VI-III cells through PI3K p110 α subunit overexpression (Fig. 4A and B). PI3K overexpression in Ras/ $I\kappa B\alpha$ -expressing Δ VI-III cells was associated with extensive tumor invasion into deep muscle fascia, whereas untreated transformed Δ VI-III cells were well encapsulated and showed no extension out of the s.c. space (Fig. 4B). Δ VI-III tumors showed

marked apoptosis, as seen by TUNEL assay (Fig. 4C and D); however, this was abrogated through PI3K overexpression. In contrast, Δ VI-III tumors continued to show proliferation as demonstrated by Ki67 expression, although it was reduced compared with normal transformed tumors and slightly increased by PI3K overexpression.

Discussion

Here, we have shown the existence of separate domains on the laminin β 3 chain which perform distinct and specific functions. The β 3 domains VI-III were shown to be dispensable for laminin-332 assembly, secretion or deposition into the matrix as well as keratinocyte migration and spreading. The β 3 domain VI proved critical for the formation of stable cell adhesion, however, this function was dispensable for SCC tumorigenesis. Deletion of either domain VI or V-III did not seem to affect the availability of integrin-binding sites on the laminin-332 molecule. This is not surprising given that the laminin β 3 short arm containing these domains is at the opposite end of the laminin-332 molecule relative to the large G domain, which is known to contain the integrin binding sites. The abnormalities in the formation of cellular SAs suggest that domain VI may interact with another hemidesmosome component or non-integrin cell surface receptor in mediating the formation of SAs. In contrast to domain VI, the β 3 domain V-III was found to be essential for SCC tumorigenesis, providing collagen VII binding which was associated with PI3K pathway activation, increased tumor invasion and protection from apoptosis. Interestingly, collagen VII null keratinocytes displayed normal adhesion but markedly impaired tumorigenic potential, whereas keratinocytes lacking laminin β 3 domain VI displayed markedly impaired adhesion but showed preservation of tumor formation and invasion. This uncoupling of adhesion from tumor progression in our studies suggests that laminin-332/collagen VII interaction promotes SCC carcinogenesis primarily through a signaling rather than an adhesive mechanism.

Our results highlight the importance of the PI3K/AKT pathway in SCC progression. The overexpression of Ras and $I\kappa B\alpha$ was not sufficient to activate this pathway in SCC tumors and cells. Rather, signals from the extracellular environment derived from laminin-332/collagen VII interaction were also needed for PI3K pathway activation and tumorigenesis. Indeed it seems that activation of the PI3K pathway, rather than adhesion, is the primary contribution of laminin-332/collagen VII interaction to SCC tumorigenesis and invasion. These studies suggest that the use of inhibitors of the PI3K pathway in the treatment of SCC warrants further investigation. In particular, our studies suggest that the targeting of the β 3 domain V-III with blocking antibodies may provide an effective method to down-regulate the PI3K pathway in SCC tumors. These studies clearly illustrate how extracellular cues of the tumor microenvironment can exert powerful effects on carcinogenesis and invasion.

Acknowledgments

Received 11/9/2006; revised 1/16/2007; accepted 2/16/2007.

Grant support: U.S. NIH grants R01 AR047223, P01 AR44012, support from Office of Research Management DVAHCS, and assistance from an Austrian APART stipend.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

1. Alam M, Ratner D. Cutaneous squamous-cell carcinoma. *N Engl J Med* 2001;344:975–83.
2. Pyke C, Salo S, Ralfkiaer E, Romer J, Dano K, Tryggvason K. Laminin-5 is a marker of invading cancer cells in some human carcinomas and is coexpressed with the receptor for urokinase plasminogen activator in budding cancer cells in colon adenocarcinomas. *Cancer Res* 1995;55:4132–9.
3. Patarroyo M, Tryggvason K, Virtanen I. Laminin isoforms in tumor invasion, angiogenesis and metastasis. *Semin Cancer Biol* 2002;12:197–207.
4. Colognato H, Yurchenco PD. Form and function: the laminin family of heterotrimers. *Dev Dyn* 2000;218:213–34.
5. Chen M, Marinkovich MP, Veis A, et al. Interactions of the amino-terminal noncollagenous (NC1) domain of type VII collagen with extracellular matrix components. A potential role in epidermal-dermal adherence in human skin. *J Biol Chem* 1997;272:14516–22.
6. Rousselle P, Keene DR, Ruggiero F, Champlaud MF, Rest M, Burgeson RE. Laminin 5 binds the NC-1 domain of type VII collagen. *J Cell Biol* 1997;138:719–28.
7. Dajee M, Lazarov M, Zhang JY, et al. NF- κ B blockade and oncogenic Ras trigger invasive human epidermal neoplasia. *Nature* 2003;421:639–43.
8. Ortiz-Urda S, Garcia J, Green CL, et al. Type VII collagen is required for Ras-driven human epidermal tumorigenesis. *Science* 2005;307:1773–6.
9. Yuspa SH, Epstein EHJ. Cancer. An anchor for tumor cell invasion. *Science* 2005;307:1727–8.
10. Marinkovich MP, Lunstrum GP, Burgeson RE. The anchoring filament protein kalinin is synthesized and secreted as a high molecular weight precursor. *J Biol Chem* 1992;267:17900–6.
11. Sasaki T, Fassler R, Hohenester E. Laminin: the crux of basement membrane assembly. *J Cell Biol* 2004;164:959–63.
12. Chen M, Marinkovich MP, Jones JC, O'Toole EA, Li YY, Woodley DT. NC1 domain of type VII collagen binds to the β 3 chain of laminin 5 via a unique subdomain within the fibronectin-like repeats. *J Invest Dermatol* 1999;112:177–83.
13. Nakashima Y, Kariya Y, Yasuda C, Miyazaki K. Regulation of cell adhesion and type VII collagen binding by the β 3 chain short arm of laminin-5: effect of its proteolytic cleavage. *J Biochem (Tokyo)* 2005;138:539–52.
14. Owaribe K, Nishizawa Y, Franke WW. Isolation and characterization of hemidesmosomes from bovine corneal epithelial cells. *Exp Cell Res* 1991;192:622–30.
15. Lammerding J, Kazarov AR, Huang H, Lee RT, Hemler ME. Tetraspanin CD151 regulates α 6 β 1 integrin adhesion strengthening. *Proc Natl Acad Sci U S A* 2003;100:7616–21.
16. Rousselle P, Lunstrum GP, Keene DR, Burgeson RE. Kalinin: an epithelium-specific basement membrane adhesion molecule that is a component of anchoring filaments. *J Cell Biol* 1991;114:567–76.
17. Robbins PB, Lin Q, Goodnough JB, et al. *In vivo* restoration of laminin 5 β 3 expression and function in junctional epidermolysis bullosa. *Proc Natl Acad Sci U S A* 2001;98:5193–8.
18. Kinsella TM, Nolan GP. Episomal vectors rapidly and stably produce high-titer recombinant retrovirus. *Hum Gene Ther* 1996;7:1405–13.
19. Deng H, Choate KA, Lin Q, Khavari PA. High-efficiency gene transfer and pharmacologic selection of genetically engineered human keratinocytes. *Biotechniques* 1998;25:274–80.
20. Holden P, Keene DR, Lunstrum GP, Bachinger HP, Horton WA. Secretion of cartilage oligomeric matrix protein is affected by the signal peptide. *J Biol Chem* 2005;280:17172–9.
21. Chudnovsky Y, Adams AE, Robbins PB, Lin Q, Khavari PA. Use of human tissue to assess the oncogenic activity of melanoma-associated mutations. *Nat Genet* 2005;37:745–9.
22. Veitch DP, Nokelainen P, McGowan KA, et al. Mammalian tolloid metalloproteinase, and not matrix metalloprotease 2 or membrane type 1 metalloprotease, processes laminin-5 in keratinocytes and skin. *J Biol Chem* 2003;278:15661–8.
23. Kleinman HK, McGarvey ML, Liotta LA, Robey PG, Tryggvason K, Martin GR. Isolation and characterization of type IV procollagen, laminin, and heparan sulfate proteoglycan from the EHS sarcoma. *Biochemistry* 1982;21:6188–93.
24. Gagnoux-Palacios L, Allegra M, Spirito F, et al. The short arm of the laminin γ 2 chain plays a pivotal role in the incorporation of laminin 5 into the extracellular matrix and in cell adhesion. *J Cell Biol* 2001;153:835–50.
25. Li J, Zhou L, Tran HT, et al. Overexpression of laminin-8 in human dermal microvascular endothelial cells promotes angiogenesis-related functions. *J Invest Dermatol* 2006;126:432–40.
26. Russell AJ, Fincher EF, Millman L, et al. α 6 β 4 integrin regulates keratinocyte chemotaxis through differential GTPase activation and antagonism of α 3 β 1 integrin. *J Cell Sci* 2003;116:3543–56.
27. Carter WG, Ryan MC, Gahr PJ. Epiligrin, a new cell adhesion ligand for integrin-3-1 in epithelial basement membranes. *Cell* 1991;65:559–610.
28. Litjens SH, de Pereda JM, Sonnenberg A. Current insights into the formation and breakdown of hemidesmosomes. *Trends Cell Biol* 2006;16:326–83.
29. Shaw LM, Rabinovitz I, Wang HH, Toker A, Mercurio AM. Activation of phosphoinositide 3-OH kinase by the α 6 β 4 integrin promotes carcinoma invasion. *Cell* 1997;91:949–60.
30. Calautti E, Li J, Saoncella S, Brissette JL, Goetinck PF. Phosphoinositide 3-kinase signaling to Akt promotes keratinocyte differentiation versus death. *J Biol Chem* 2005;280:32856–65.

Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

A Laminin-Collagen Complex Drives Human Epidermal Carcinogenesis through Phosphoinositol-3-Kinase Activation

Elizabeth A. Waterman, Noriyasu Sakai, Ngon T. Nguyen, et al.

Cancer Res 2007;67:4264-4270.

Updated version	Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/67/9/4264
Supplementary Material	Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2007/04/30/67.9.4264.DC1

Cited articles	This article cites 30 articles, 15 of which you can access for free at: http://cancerres.aacrjournals.org/content/67/9/4264.full#ref-list-1
Citing articles	This article has been cited by 9 HighWire-hosted articles. Access the articles at: http://cancerres.aacrjournals.org/content/67/9/4264.full#related-urls

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org .
Permissions	To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/67/9/4264 . Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.